Effects of temperature on slow and fast inactivation of rat skeletal muscle Na⁺ channels

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Ruff, Robert L. Effects of temperature on slow and fast inactivation of rat skeletal muscle Na⁺ channels. Am. J. Physiol. 277 (Cell Physiol. 46): C937–C947, 1999.—Patch-clamp studies of mammalian skeletal muscle Na⁺ channels are commonly done at subphysiological temperatures, usually room temperature. However, at subphysiological temperatures, most Na⁺ channels are inactivated at the cell resting potential. This study examined the effects of temperature on fast and slow inactivation of Na⁺ channels to determine if temperature changed the fraction of Na⁺ channels that were excitable at resting potential. The loose patch voltage clamp recorded Na⁺ currents (Iₙa) in vitro at 19, 25, 31, and 37°C from the sarcolemma of rat type IIb fast-twitch omohyoid skeletal muscle fibers. Temperature affected the fraction of Na⁺ channels that were excitable at the resting potential. At 19°C, only 30% of channels were excitable at the resting potential. In contrast, at 37°C, 93% of Na⁺ channels were excitable at the resting potential. Temperature did not alter the resting potential or the voltage dependencies of activation or fast inactivation. Iₙa available at the resting potential increased with temperature because the steady-state voltage dependence of slow inactivation shifted in a depolarizing direction with increasing temperature. The membrane potential at which half of the Na⁺ channels were in the slow inactivated state was shifted by +16 mV at 37°C compared with 19°C. Consequently, the low availability of excitable Na⁺ channels at subphysiological temperatures resulted from channels being in the slow, inactivated state at the resting potential.

mammalian skeletal muscle; sodium channel; sodium current; fast inactivation; slow inactivation; paramyotonia congenita; hyperkalemic periodic paralysis

MAMMALIAN SKELETAL MUSCLE and other excitable tissues have two inactivation processes with different kinetics and voltage dependencies (1, 2, 14, 21, 34–36, 38, 39, 41). Fast inactivation closes channels on a millisecond time scale, whereas slow inactivation takes seconds to minutes. In rat and human skeletal muscle, fast inactivation helps to terminate the action potential (1, 34, 36). Slow inactivation is too slow to affect action potential termination. However, in mammalian skeletal muscle, slow inactivation operates at more negative potentials than fast inactivation, so that the distribution of channels between the closed and slow inactivated state regulates the number of excitable Na⁺ channels as a function of the membrane potential (32, 35, 36, 38, 39, 41). Slow inactivation changes the number of excitable channels but does not change the single-channel conductance or single-channel open time (32). Physiological studies suggest that fast and slow inactivated states are distinct (5, 30, 32, 42, 45). Na⁺ channel mutations can independently change fast or slow inactivation (10, 16, 17, 46). Therefore, the slow and fast inactivated states probably represent distinct Na⁺ channel conformations. Although Na⁺ channels may undergo slow inactivation from either open, closed, or fast-inactivated states (32), the rate of development of slow inactivation or recovery from slow inactivation and the completeness of slow inactivation may be influenced by the conformations of either the activation or fast inactivation gates (13, 43).

Voltage-gated Na⁺ channels are responsible for the rising phase and subsequent propagation of action potentials in muscle, nerve, and secretory tissues. One of the important determinants of membrane excitability is the availability of Na⁺ channels. The density of excitable Na⁺ channels depends on the density of channels in the membrane and the fraction of channels that are excitable (34). Most patch-clamp studies of Na⁺ channels are performed at room temperature. A consistent and puzzling finding in room temperature studies of cloned Na⁺ channels expressed in a variety of cell systems is that a low fraction, 50% or less, of Na⁺ channels are excitable at membrane potentials of −80 mV to −90 mV (20, 26, 43, 47). Similarly, in vitro studies performed at room temperature on fast-twitch skeletal muscle fibers from rats (32, 34, 35, 41), rabbits (21), or humans (34, 38, 39) indicate that more than 50% of Na⁺ channels are inexcitable at the resting potential due to Na⁺ channel slow inactivation. In vivo patch-clamp data on rat fast-twitch skeletal muscle fibers indicate that at physiological temperature most Na⁺ channels are excitable at the cell resting potential. The amplitudes of Na⁺ current density (Iₙa) at the resting potential of rat fast-twitch fibers studied in vivo (37) are similar to the maximum values of Iₙa obtained from in vitro recordings at 19°C (34). However, to obtain the maximum value of Iₙa for in vitro studies, the membrane must be sufficiently hyperpolarized for a prolonged time to recover Na⁺ channels from slow inactivation (34). Consequently, a physiologically unreasonably large fraction of Na⁺ channels are inexcitable at room temperature because they are in the slow inactivated state.

Temperature could influence the slow inactivation process in several ways. In some studies of cloned Na⁺ channels studied in different expression systems, only 80–85% of Na⁺ channels were subject to slow inactiva-
Elevated temperature could increase the fraction of excitable Na⁺ channels by reducing the fraction of Na⁺ channels that can be slow inactivated. Alternatively, temperature could change the operational voltage range for slow inactivation. The high proportion of slow inactivated Na⁺ channels at the resting potential found at subphysiological temperatures could result if at low temperatures the slow inactivation-membrane potential relationship was shifted in a hyperpolarized direction.

This study used a loose patch voltage clamp to examine fast and slow inactivation of Iₙa in fast-twitch, type IIb, rat skeletal muscle fibers at temperatures from 19°C to 37°C. The fraction of Na⁺ channels that were excitable at the resting potential increased with temperature. This study examined the hypothesis of whether temperature altered the fraction of Na⁺ channels that were excitable at the resting potential by changing the steady-state voltage dependence of slow inactivation.

**METHODS**

Male Wistar rats (290–320 g) were anesthetized with intraperitoneally injected pentobarbital, omohyoid muscles were removed, and the rats were killed by pentobarbital overdose.

Tissue handling. Muscle fibers were dissected and studied in a solution composed of 19 parts Tyrode solution (95% by volume) and 1 part rat serum (5% by volume). The presence of 5% rat serum in the bathing solution greatly increased the survival time for muscle fibers at 31 and 37°C. In bathing solution with 5% rat serum, omohyoid muscle fibers maintained resting potentials of less than ~90 mV for more than 1 h. The rat serum was obtained by exsanguinating rats, allowing the removed blood to clot and collecting the clear serum. Serum was discarded if any visible evidence of hemolysis was present. Serum was centrifuged at 5,000 rpm to remove any cellular particulate material. The supernatant serum was filtered through a 0.01-µm Millipore filter and stored at ~70°C. Tyrode solution contained (in mM) 135 NaCl, 3.5 KCl, 1 MgCl₂, 6 CaCl₂, 10 HEPES and 10 glucose. Solutions were vigorously gassed with O₂, and the pH was 7.4.

Dissections were performed at 19°C. Surface connective tissue was removed as completely as possible by a combination of dissection and collagenase treatment (32, 38, 39). The dissection began after the muscle had been in oxygenated, circulated Tyrode solution containing 2 mg/ml of collagenase (Sigma type I) and 1 mg/ml of bovine albumin (Sigma fraction V) for 45 min. In the later stages of dissection, the collagenase-containing solution was perfused only over the middle of the fibers so that the tendon attachments remained intact. The enzymatic treatment enabled dissection of the muscle to a preparation that was only a few fibers thick and greatly improved the quality of the seals between the voltage-clamp pipettes and the sarcolemma. Before current recordings were made, the collagenase was diligently washed from the bathing chamber. Iₙa recordings were made >200 µm from the endplate region to avoid the region of high Na⁺ channel density near the endplate (38, 39).

Temperature regulation. Experiments were performed at 19, 25, 31, and 37°C. The recording chamber had three thermistors that monitored bath temperature. The three temperature values had to all be within 0.2°C of the target temperature for an experiment to proceed. The temperature of the recording chamber was controlled with a Peltier device (Cambion, Cambridge, MA) with a direct current feedback controller. The chamber used for recording Iₙa was modified to reduce the exposed solution-air interface to 5 mm × 7 mm. In addition, the chamber was perfused by one of four reservoirs of bathing solution. Each reservoir was maintained at one of the target temperatures by a surrounding temperature-controlled water bath. The temperature of the recording chamber could be changed within 10 s. A muscle preparation was kept at a recording temperature for at least 5 min before recording Iₙa or membrane potentials.

Loose patch voltage clamp. Technical details of the loose patch voltage clamp technique used to measure Iₙa were previously described (32, 38, 39). With the bathing solution described in Tissue handling, the resistive seals between the cleaned muscle membranes and pipettes were >20 MΩ at all temperatures studied. The high seal resistance reduced the fraction of membrane current that passed across the seal resistance rather than through the pipette and improved the frequency resolution and response time of the patch clamp. The fraction of membrane current lost across the seal was corrected for by analog and digital compensation. Micropipettes had tip diameters after fire polishing of ~10 µm and resistances of 200–300 KΩ. These sizes of pipettes were chosen to permit sampling from a sufficiently large patch of membrane to reduce local variations in Iₙa density and yet not to stimulate too large a current so that voltage control of the cell was maintained. The pipettes were coated with a double layer of Sylgard (Dow Corning 184, Midland, MI) to within 100 µm of the tip to reduce capacitive coupling between the bath and pipette. Minimal suction was applied to the loose patch micropipettes to avoid the formation of membrane blebs (24, 38).

Measurement of membrane current with the loose patch. A potential applied to the micropipette changed the transmembrane potential of the small patch of membrane under the pipette. Analog and digital corrections compensated for the current that flowed across the seal between the pipette and the sarcolemma so that the transmembrane potential was controlled and the transmembrane current could be measured (35, 38, 39, 41). The maximum inward Iₙa from a patch of membrane at a given holding potential, Iₙa,max, was determined by a group of six depolarizing test pulses. Test pulses were 4 ms long at 19, 25, and 31°C and 2 ms long at 37°C. Each test pulse was preceded by a 20-ms, 50-mV hyperpolarizing prepulse, relative to the holding potential, to remove fast inactivation of Iₙa. In some experiments, the 20-ms hyperpolarizing prepulses to remove fast inactivation were scaled so that all prepulses were to ~120 mV. The depolarization of the test pulses were incremented in 6-mV steps. Voltages of the test pulses were chosen to bracket the membrane potential that elicited Iₙa,max, which is called Vₙa,max. Details of the pulse protocols used to measure Iₙa and the calculations of Iₙa,max and Vₙa,max from the six pulse protocols were previously described (38, 39). Iₙa,max was measured every 15 s or every 30 s to assay the state of slow inactivation of macroscopic Iₙa.

Measurement of the voltage dependence of fast inactivation. The steady-state voltage dependence of fast inactivation was studied by applying 20-ms conditioning prepulses that were immediately followed by 4-ms depolarizing test pulses to about Vₙa,max at 19, 25, and 31°C (35, 36, 38, 39, 41). At 37°C, the test pulses were 2 ms long. A prepulse duration of 20 ms was sufficient long to study fast inactivation in rat skeletal muscle fibers at the membrane potentials examined in this study (35, 36, 38, 39, 41). The steady-state voltage dependence of fast inactivation, the “h” curve, was described by a
Boltzmann distribution

\[ h = \frac{I_{Na}(V) - I_{Na}\text{max}}{I_{Na}(V) - I_{Na}\text{max}} = \frac{1}{1 + \exp[(V_m - V_{1/2})/A_h]} \]  

where \( I_{Na} / I_{Na}\text{max} \) is the relative amplitude of \( I_{Na} \) during a test pulse, \( V_m \) is the membrane potential during a prepulse, \( V_{1/2} \) is the potential of the prepulse that inactivates half the Na\(^+\) channels by the fast inactivation process, and \( A_h \) is a parameter determining the steepness of the \( h \)-membrane potential relationship.

Slow inactivation. Slow inactivation was studied by measuring the size and time course of the change in \( I_{Na}\text{max} \) after the holding potential changed. The steady-state values of \( I_{Na}\text{max} \) at a given membrane potential and the time constant of the change in current density following a change in potential were obtained by a least squares fit of the following function to \( I_{Na}\text{max} \)

\[ I_{Na}(t) = I_{Na}\text{final} - [(I_{Na}\text{final} - I_{Na}\text{initial})\exp(-t/\tau)] \]  

where \( I_{Na}\text{initial} \) is the initial \( I_{Na}\text{max} \), \( I_{Na}\text{final} \) is the steady-state \( I_{Na}\text{max} \) after changing the potential, \( I_{Na}(t) \) is the current density at each time, and \( \tau \) is the time constant. Equation 2 was also used to determine the time constant for development of fast inactivation, \( \tau_h \), from records of \( I_{Na} \).

Due to the time it took for slow inactivation to reach steady state after a change in membrane potential, the voltage sensitivity of slow inactivation could not be completely described for a single fiber. Therefore, the holding potential at which the maximal \( I_{Na}\text{max} \) was obtained and the relative value of \( I_{Na}\text{max} \) at other holding potentials for fibers within a given group of fibers were plotted together. The smooth curves were the least squares fits of a Boltzmann distribution to the data

\[ s = \frac{I_{Na\text{max}}}{\text{maximal} I_{Na\text{max}}} = \frac{1}{1 + \exp[(V_m - V_{s1/2})/A_s]} \]  

where \( s \) is the steady-state slow inactivation of \( I_{Na} \), \( I_{Na\text{max}} \) is the steady-state \( I_{Na\text{max}} \) obtained at a given holding potential relative to the maximal \( I_{Na\text{max}} \) that could be obtained when slow inactivation was completely removed, \( V_{s1/2} \) is the potential at which 50% of Na\(^+\) channels were closed due to slow inactivation, and \( A_s \) describes the steepness of the voltage dependence of slow inactivation.

Membrane Na\(^+\) conductance. Membrane Na\(^+\) conductance was calculated from a least squares of the following equation to the data

\[ G(V_m) = I_{pK}(V_m)/(V_m - E_{rev}) \]  

where \( G(V_m) \) is the Na\(^+\) conductance as a function of membrane potential, \( I_{pK}(V_m) \) is the peak inward \( I_{Na} \) produced by a depolarizing test potential to \( V_m \), and \( E_{rev} \) is the reversal potential for \( I_{Na} \). The membrane potential at which \( G(V_m) \) was half-maximal was called \( V_{G1/2} \). \( E_{rev} \) was calculated as the zero current intercept for current-voltage relationships obtained from current traces such as those shown in Fig. 1. For the current traces in Fig. 1, \( E_{rev} \) was +49 mV at 19°C and +47 mV at 37°C.

Resting potentials. Resting potentials were measured with an intracellular microelectrode filled with 3 M KCl at a distance of 0.1–0.15 mm from the patch electrodes. Resting potential was measured for each cell after completing \( I_{Na} \) recordings. \( V_{G1/2} \) was measured before and after impalement with the voltage electrode to determine the depolarization produced by the impalement. The actual resting potential was the potential measured by the voltage electrode minus the depolarization associated with the impalement (35, 38, 39, 41). The membrane potential throughout an experiment was determined from three factors: 1) the directly measured membrane potential at the end of an experiment, 2) the effect of temperature on the resting potential (see Table 1), and 3) changes in \( V_{G1/2} \), which were used to determine shifts in the membrane potential over time for experiments at a single temperature. Current recordings were stopped if the compensation of the resting membrane potential for different temperatures combined with changes in \( V_{G1/2} \) showed that the membrane potential had changed by more than 5 mV from the beginning of an experiment.

Histochemical fiber type. Histochemical fiber type was determined at the completion of current recordings, as previously described, from analysis of a segment of the fiber that \( I_{Na} \) was recorded from (38, 39).

Statistical analysis. Data were analyzed with ANOVA using two-tailed tests with a set at 0.05 (38, 39). Curve fitting was performed using a commercial product, SigmaPlot (Jandel Scientific, San Rafael, CA). Values are shown as means ± SE.

**RESULTS**

This study examined \( I_{Na} \) from rat omohyoid type IIb skeletal muscle fibers at 19, 25, 31, and 37°C. The effects of temperature on the voltage dependencies of activation and fast inactivation of \( I_{Na} \) were studied in 12 cells at each of the four study temperatures. Seven cells were studied at three temperatures, and 2 cells were studied at only two temperatures. \( I_{Na} \) measurements were made on 18 cells at 19°C, 19 cells at 25°C, 19 cells at 31°C, and 18 cells at 37°C. The voltage dependence and kinetics of slow inactivation were determined from 15 fibers at 19°C, 13 fibers at 25°C, 15 fibers at 31°C, and 14 fibers at 37°C.

Effects of temperature on resting membrane potential. The resting potential of a fiber, determined by impaling the cell with an intracellular microelectrode, could be measured only at the end of a current recording session because cells studied with the loose patch clamp would depolarize after the membrane was impaled (35, 41). For studies at a single temperature, changes in \( V_{G1/2} \) were used to measure alterations of the cell resting potential during the course of an experiment (32). Because it was not known if \( V_{G1/2} \) varied with temperature, the strategy used to determine the membrane potential of a cell during the course of an experiment

<table>
<thead>
<tr>
<th>Temperature</th>
<th>RP, mV</th>
<th>( V_{G1/2} ), mV</th>
<th>( V_{G1/2} ), mV</th>
<th>( A_s ), mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>19°C</td>
<td>-97.9 ± 1.0</td>
<td>-35.9 ± 0.6</td>
<td>-75.1 ± 1.9</td>
<td>5.10 ± 0.09</td>
</tr>
<tr>
<td>25°C</td>
<td>-98.3 ± 1.1</td>
<td>-35.8 ± 0.6</td>
<td>-75.9 ± 2.2</td>
<td>5.13 ± 0.09</td>
</tr>
<tr>
<td>31°C</td>
<td>-99.1 ± 0.9</td>
<td>-35.5 ± 0.6</td>
<td>-77.2 ± 2.2</td>
<td>5.14 ± 0.09</td>
</tr>
<tr>
<td>37°C</td>
<td>-99.9 ± 1.1</td>
<td>-35.1 ± 0.8</td>
<td>-78.5 ± 2.4</td>
<td>5.20 ± 0.11</td>
</tr>
</tbody>
</table>

Values are means ± SE. Resting membrane potential (RP) measurements were made from 40 or more cells at each temperature. For RP measurements, cells were studied at only 1 temperature. For the other measurements, cells were studied at 2 or more temperatures, with 18 cells studied at 19°C, 19 cells studied at 25°C, 19 cells studied at 31°C, and 18 cells studied at 37°C. \( V_{G1/2} \) membrane potential at which conductance was half maximal; \( V_{G1/2} \), potential that inactivates half the Na\(^+\) channels by fast inactivation of Na\(^+\) current; \( A_s \), parameter determining the steepness of the steady-state fast inactivation of Na\(^+\) current-membrane potential relationship.
was modified when the cell was studied at several temperatures. First, this study determined the effect of temperature on the resting potential of the omohyoid muscle fibers. Second, to ensure that changes in the voltage dependence of $I_{Na}$ at different temperatures were reversible and reflected only the effects of temperature, $I_{Na}$ data were accepted only from fibers that were studied at two or more temperatures when at least one temperature was studied twice and the voltage dependencies of $I_{Na}$ values for recordings at the same temperature were comparable.

This study determined the effect of temperature on the resting membrane potential of cells by measuring the resting potential of 40 or more fibers at each study temperature. Table 1 shows a tendency for fibers to increase in resting potential of 40 or more fibers at each study temperature during the course of an experiment using the following steps: 1) the membrane potential was determined at the end of an experiment by direct measurement, 2) changes in the value of $V_{h}$ were used to determine changes in the resting potential during portions of an experiment performed at a specific temperature, and 3) the differences among the resting potentials shown in Table 1 were used to determine changes in the membrane potential associated with changing the experiment’s temperature.

Temperature effects on activation and fast inactivation of $I_{Na}$. Figure 1 shows inward $I_{Na}$ traces generated at 19 and 37°C from the same type IIB fiber. On the basis of the resting potential values shown in Table 1, the membrane hyperpolarized by 2 mV when the temperature was increased from 19 to 37°C. At 19°C, the inward currents were stimulated by depolarizing pulses from $-55$ mV to $+35$ mV in 10-mV increments. At 37°C, the inward $I_{Na}$ traces were stimulated by depolarizing pulses from $-57$ mV to $+33$ mV.

Currents activated faster, and fast inactivation developed more rapidly at 37°C compared with at 19°C. Figure 2 shows the voltage dependence for the apparent rate constant for development of fast inactivation, $1/\tau_{in}$, for the current traces shown in Fig. 1. Over the voltage ranges shown in Fig. 2, $1/\tau_{in}$ varied linearly with voltage. The slope of the $1/\tau_{in}$-voltage relationship was more than 10-fold steeper at 37°C. At 19°C, the slope of the $1/\tau_{in}$-voltage relationship was $0.0267 \text{ mV}^{-1} \text{·msec}^{-1}$ and at 37°C the slope was $0.300 \text{ mV}^{-1} \text{·msec}^{-1}$. The apparent rate of development of fast inactivation at 0 mV ($1/\tau_{0in}$) was 15-fold faster at 37°C compared with that at 19°C.

Figure 3 shows the temperature dependence of $\tau_{in}$, as an Arrhenius plot, for 12 cells studied at 19, 25, 31, and 37°C. In Fig. 3, the logarithm of the mean value of $\tau_{in}$ was plotted against temperature. The linear decline of the logarithm of $\tau_{in}$ with temperature corresponded to a $Q_{10}$ value of 4.13 for $1/\tau_{in}$ over the temperature range of 19 to 37°C.

Temperature did not appreciably alter the voltage dependencies of activation or fast inactivation. Figure 4 shows the voltage dependences of activation and fast inactivation at 19 and 37°C for the 13 cells studied at both temperatures. $V_{1/2}$ occurred at $-36.0 \pm 0.7$ mV at 19°C and at $-35.2 \pm 0.8$ mV at 37°C. The voltage dependences of fast inactivation were similar at 19 and 37°C. $V_{1/2}$ was $-75.1 \pm 1.8$ mV at 19°C and $-78.5 \pm 2.1$ mV at 37°C. $A_{n}$ values were also similar ($5.1 \pm 0.8$ mV.
at 19°C and 5.2 ± 0.7 mV at 37°C. Table 1 shows the values for $V_{h1/2}$, $A_n$, and $V_{G1/2}$ for the 21 cells that were studied at two or more temperatures. The values of $V_{h1/2}$, $A_n$, and $V_{G1/2}$ in Table 1 were not significantly different at 19°C compared with those at 37°C and were not significantly different when values at 19°C were compared with those at any other temperature.

Temperature increased the amount of $I_{Na}$ available at the resting potential. In contrast to temperature not affecting the voltage dependencies of activation and fast inactivation, temperature appreciably altered the amount of $I_{Na}$ elicited by depolarizations from the resting potential. The maximum inward current at the resting potential ($I_{NaRP}$) increased with temperature. In Fig. 1, at 19°C, $I_{NaRP}$ was 9.4 mA/cm² at −103 mV and, at 37°C, $I_{NaRP}$ was 19.2 mA/cm² at −105 mV. The increase in $I_{NaRP}$ could have resulted from a shift in the voltage dependence of slow inactivation or a reduction in the fraction of channels affected by slow inactivation. Either alteration in slow inactivation would have reduced the number of channels at the resting potential that were slow inactivated at higher temperature. Alternatively, increased temperature could have unmasked hidden channels or increased the maximum probability that a channel would open with depolarization ($P_o$). If temperature acted by shifting the voltage dependence of slow inactivation or by reducing the fraction of channels susceptible to slow inactivation, then the density of excitabale $Na^+$ channels measured after prolonged hyperpolarizations that removed slow inactivation should be comparable at different temperatures. If increased temperature unmasked $Na^+$ channels or increased $P_o$ then the maximum density of open channels in response to a depolarizing pulse would be larger at 37°C compared with at 19°C. $I_{Namax}$ provided an experimental assay of the density of open channels.

The single-channel conductance should increase with a $Q_10$ value of −1.3 (18, 19, 23). Consequently, if temperature did not change the density of excitable channels, then $I_{Namax}$ should have increased −1.6-fold from 19 to 37°C, corresponding to the $Q_10$ value of 1.3. For the cell shown in Fig. 1, $I_{Namax}$ after slow inactivation was removed was 17.3 mA/cm² at 19°C and 20.3 mA/cm² at 37°C. The maximal $I_{Namax}$ increased only 1.2-fold in response to a 18°C increase in temperature for the cell shown in Fig. 1. Table 2 shows that maximal $I_{Namax}$ increased slightly with temperature. The temperature-dependent increase in maximal $I_{Namax}$ shown in Table 2 corresponded to a $Q_10$ of 1.21. Therefore, temperature did not increase the maximum density of open channels produced by a depolarizing pulse. Consequently, the effect of temperature on $I_{Namax}$ was probably mediated by an effect of temperature on slow inactivation and not the result of unmasking $Na^+$ channels or increasing $P_o$.

Table 2 shows that the fraction of $I_{Na}$ available at the resting potential compared with the maximal value of $I_{Na}$ with slow inactivation removed ($I_{NaRP}$/maximal $I_{Namax}$) increased with temperature. At 37°C, 93% of $Na^+$ channels were excitabale at the resting potential compared with only 30% of channels being excitabale at 19°C.

Temperature changed the voltage dependence of slow inactivation of $I_{Na}$. To determine if temperature changed the voltage dependence of slow inactivation or altered the fraction of $Na^+$ channels that were susceptible to slow inactivation, slow inactivation was studied at each of the study temperatures. Because of the time required to study slow inactivation, it was not practical to study slow inactivation in the same cell at different temperatures. Table 2 shows the number of cells evaluated at each temperature at which slow inactivation was studied. Figure 5 shows the development of and recovery from slow inactivation of $I_{Na}$ for a fiber at 19°C. Note that $I_{Namax}$ at −100 mV was only 60% of the maximal value of $I_{Namax}$ at −150 mV when slow inactivation was removed. Figure 6 shows the development of and recovery from slow inactivation of $I_{Na}$ for a fiber at...
Table 2. Effects of temperature on the peak $I_{\text{Na}}$ available at RP and the steady-state voltage dependence of slow inactivation

<table>
<thead>
<tr>
<th>Temperature</th>
<th>n</th>
<th>RP, mV</th>
<th>$I_{\text{Na}}$, mA·cm$^{-2}$</th>
<th>$V_{1/2}$, mV</th>
<th>$A_s$, mV</th>
<th>$I_{\text{Na}}$/maximal $I_{\text{Na}}$max</th>
</tr>
</thead>
<tbody>
<tr>
<td>19°C</td>
<td>15</td>
<td>-99.2 ± 1.8</td>
<td>18.3 ± 2.1</td>
<td>104 ± 2.1</td>
<td>5.71 ± 0.79</td>
<td>0.301 ± 0.09</td>
</tr>
<tr>
<td>25°C</td>
<td>13</td>
<td>-100.1 ± 1.9</td>
<td>20.4 ± 2.2</td>
<td>-99.5 ± 2.3</td>
<td>5.53 ± 0.89</td>
<td>0.527 ± 0.06</td>
</tr>
<tr>
<td>31°C</td>
<td>15</td>
<td>-101.2 ± 1.7</td>
<td>22.6 ± 2.3</td>
<td>-92.1 ± 2.5</td>
<td>5.54 ± 0.82</td>
<td>0.842 ± 0.04</td>
</tr>
<tr>
<td>37°C</td>
<td>14</td>
<td>-102.1 ± 1.9</td>
<td>25.8 ± 2.5</td>
<td>-87.9 ± 2.4</td>
<td>5.42 ± 0.91</td>
<td>0.932 ± 0.03</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of fibers studied at each temperature. Maximal $I_{\text{Na}}$, maximum inward Na$^+$ current ($I_{\text{Na}}$) that was obtained after slow inactivation was removed by hyperpolarizing the membrane; $I_{\text{Na}}$/maximal $I_{\text{Na}}$max, peak inward $I_{\text{Na}}$ at the resting potential; $V_{1/2}$, potential at which 50% of Na$^+$ channels were closed due to slow inactivation; $A_s$, parameter determining steepness of the steady-state slow inactivation of $I_{\text{Na}}$-membrane potential relationship.

37°C. For this fiber, $I_{\text{Na}}$/maximal at the resting potential of −105 mV was 95% of the maximal value of $I_{\text{Na}}$/maximal and $I_{\text{Na}}$/maximal at −90 mV was 55% of the maximal value of $I_{\text{Na}}$/maximal. Note that, in Fig. 5, slow inactivation reduced $I_{\text{Na}}$/maximal to <1% of the maximal value of $I_{\text{Na}}$/maximal and that, in Fig. 6, slow inactivation reduced $I_{\text{Na}}$/maximal to <5% of the maximal value of $I_{\text{Na}}$/maximal. The data in Figs. 5 and 6 show that all or almost all Na$^+$ channels were susceptible to slow inactivation.

For the experiments shown in Figs. 5 and 6, $I_{\text{Na}}$/maximal was measured by removing fast inactivation using 20-ms-long hyperpolarizing prepulses. The prepulses amplitudes were 50 mV hyperpolarized relative to the holding potential. Four different cells were studied at 19 and 37°C using a different protocol in which the hyperpolarizing prepulses were set to −120 mV. The amplitudes of the prepulses needed to bring the membrane potential to −120 mV during these four experiments were determined from the estimated potential of the membrane patch. The potential of the membrane patch was estimated by comparing the change in the tip of the patch pipette that was needed to fast inactivate 50% of the channels with the values of $V_{1/2}$ shown in Table 1. The data on the development of and recovery from slow inactivation obtained from the four cells studied at 19 and 37°C using prepulses to −120 mV to remove fast inactivation were similar to the data shown in Figs. 5 and 6.

The voltage dependence of slow inactivation was determined by plotting the steady-state values for $I_{\text{Na}}$/maximal vs. membrane potential for all of the fibers studied at a given temperature. Figure 7 shows the voltage dependence of $s_1$ for 15 fibers at 19°C and 14 fibers at 37°C. At both temperatures, slow inactivation could eliminate $I_{\text{Na}}$/maximal, which indicates that all of the Na$^+$ channels were susceptible to slow inactivation. $V_{1/2}$ occurred at −104.0 ± 2.1 mV at 19°C and at −87.9 ± 2.7 mV at 37°C ($P < 0.001$). The slopes of the steady-state slow inactivation curves were similar with $A_s = 5.7 ± 0.8$ mV at 19°C and 5.4 ± 0.9 mV at 37°C. Table 2 shows the values of $V_{1/2}$ and $A_s$ at 19, 25, 31, and 37°C. Figure 8 shows that $V_{1/2}$ shifted linearly toward more positive potentials with increasing temperature. $V_{1/2}$ increased by 16.1 mV from 19°C to 37°C. The slope of the temperature dependence of $V_{1/2}$ was 0.894 mV·°C$^{-1}$.

Figures 4 and 7 show that, despite the positive shift in $V_{1/2}$ with increasing temperature, $V_{1/2}$ was more negative than $V_{1/2}$ from 19°C to 37°C. At 19°C, $V_{1/2} - V_{1/2}$ was −29.2 mV, and, at 37°C, $V_{1/2} - V_{1/2}$ was −9.6 mV.
Temperature accelerated the kinetics of slow inactivation of \( I_{Na} \).

Figures 5 and 6 show that slow inactivation developed and recovered more rapidly at 37°C compared with at 19°C. Figure 9 plots \( t_s \) vs. membrane potential for slow inactivation at 19°C and 37°C. The configuration of the \( t_s \)-voltage relationship changed in two ways with temperature. First, \( t_s \) decreased with increasing temperature. Second, the maximal value of \( t_s \) (\( t_{s_{\text{max}}} \)) shifted in a depolarizing direction with increasing temperature. To determine \( t_{s_{\text{max}}} \) at each of the study temperatures, a fourth-degree polynomial was fitted to the \( t_s \)-membrane potential relationships, such as are shown in Fig. 9. The \( t_{s_{\text{max}}} \) occurred at about the same voltage as \( V_{s_{1/2}} \). At 19°C, \( t_{s_{\text{max}}} \) was 10-fold greater than that at 37°C. Figure 10, an Arrhenius plot of \( t_{s_{\text{max}}} \), shows the linear decline in the logarithm of \( t_{s_{\text{max}}} \) with increasing temperature. The \( Q_{10} \) for the decline of \( t_{s_{\text{max}}} \) with temperature over the range from 19°C to 37°C was 3.60.

**DISCUSSION**

This study demonstrated that temperature profoundly affected the fraction of \( Na^+ \) channels that were excitable at the resting potential in fast-twitch mammalian skeletal muscle. As shown in Table 2, only 30% of \( Na^+ \) channels were excitable at the resting potential at 19°C compared with 93% of channels being excitable at 37°C. The reason more \( Na^+ \) channels were excitable at the resting potential was that the midpoint of the \( s_s \)-membrane potential relationship shifted by 16.1 mV from \( V_{s_{1/2}} = -104.0 \pm 2.1 \) mV at 19°C and \( -87.9 \pm 2.7 \) mV at 37°C (\( P < 0.001 \)). Parameter determining the steepness of the \( s_s \)-membrane potential relationship was 5.7 \( \pm 0.8 \) mV at 19°C and 5.4 \( \pm 0.9 \) mV at 37°C.

Fig. 7. Voltage dependence of steady-state slow inactivation for 15 fibers at 19°C and 14 fibers at 37°C. Smooth curves are least squares fits of Eq. 3 (see text) to the data points for 19°C (dashed line) and 37°C (solid line). Potential at which 50% of \( Na^+ \) channels were closed due to slow inactivation (\( V_{s_{1/2}} \)) was \( -104.0 \pm 2.1 \) mV at 19°C and \( -87.9 \pm 2.7 \) mV at 37°C (\( P < 0.001 \)). Parameter determining the steepness of the \( s_s \)-membrane potential relationship was 5.7 \( \pm 0.8 \) mV at 19°C and 5.4 \( \pm 0.9 \) mV at 37°C.

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The kinetics of fast inactivation (Figs. 2 and 3) and slow inactivation (Figs. 9 and 10) became faster with increased temperature. The absolute values and temperature dependence of $\tau_{\text{h},\text{Na}^{+}}$ found in this study were similar to the values found for cloned wild-type rat skeletal muscle Na$^{+}$ channels (rSkM1, µ1) expressed in human embryonic kidney (HEK) cells (15), wild-type human skeletal muscle Na$^{+}$ channels (hSkM1) expressed in the tsA201 line of transformed human kidney cells (8), and hSkM1 Na$^{+}$ channels in human myoballs (28). The $Q_{10}$ for $\tau_{\text{h},\text{Na}^{+}}$ was 4.13 in this study compared with 3.34 for rSkM1 Na$^{+}$ channels studied between 12 and 37°C (15), 3.2 for the hSkM1 Na$^{+}$ channels expressed in tsA201 cells studied between 14 and 30°C (8), and 3.6 for hSkM1 Na$^{+}$ channels in myoballs studied between 10 and 37°C (28).

This study found only slight effects of temperature on the voltage dependencies of activation of conductance and $h_{\text{Na}}$ (Fig. 4, Table 1). Prior studies on mammalian skeletal muscle, cardiac muscle, and peripheral nerve Na$^{+}$ channels found variable effects of temperature on the voltage dependencies of activation and fast inactivation. Human myoballs express both hSkM1 and hSkM2 Na$^{+}$ channels (28). The hSkM2 channel is relatively resistant to TTX, expressed on immature and denervated skeletal muscle cells, and is the same at the most commonly found cardiac Na$^{+}$ channel (20). The voltage dependencies of activation and fast inactivation did not vary over the temperature range from 10 to 37°C for the hSkM1 Na$^{+}$ channels in myoballs. In contrast, for the hSkM2 Na$^{+}$ channels in myoballs, fast inactivation shifted in a hyperpolarizing direction with reduced temperature, but the voltage dependence of activation did not vary with temperature (28). In guinea pig ventricular myocytes, fast inactivation of $I_{\text{Na}}$ shifted in a hyperpolarizing direction, as temperature was reduced from 36 to 15°C (25). In sheep cardiac Purkinje fibers, temperature did not appreciably alter the voltage dependence of peak Na$^{+}$ permeability over a range from 10 to 24°C or $h_{\text{Na}}$ over a range from 16 to 26°C (9). In rat peripheral nerve nodes of Ranvier, the voltage dependencies of activation and fast inactivation did not change with temperature between 22 and 37°C (40). In summary, the lack of effect of temperature on the voltage dependencies of activation and fast inactivation found in this study is consistent with prior findings for hSkM1 Na$^{+}$ channels in human myoballs and Na$^{+}$ channels in rat peripheral myelinated nerve fibers.

Prior studies on mammalian skeletal muscle and cardiac Na$^{+}$ channels suggested that reducing temperature might decrease the fraction of excitable Na$^{+}$ channels at the cell resting potential. Resealed human skeletal muscle fiber segments studied at 21°C had to be maintained at holding potential of -110 mV for 2-5 min to recover Na$^{+}$ channels from slow inactivation (14). In guinea pig cardiac ventricular myocytes, the maximum Na$^{+}$ conductance elicited by depolarizing steps from the resting potential increased by 69% at 25°C compared with 15°C and peak Na$^{+}$ conductance was 41% larger at 35°C compared with that at 25°C (25). In sheep cardiac Purkinje fibers, the amplitude of peak inward $I_{\text{Na}}$ elicited by depolarizations from the resting potential increased by 50% at 26°C compared with at 16°C (9). Dudel and Rüdel (11) found that very long duration hyperpolarizing prepulses were required to recover $I_{\text{Na}}$ for recordings at 10°C and below. The findings described in the above experiments on mammalian cardiac and skeletal muscle cells are consistent with increased inactivation of $I_{\text{Na}}$ at lower temperatures.

In this study, the s.-membrane potential relationship was shifted in hyperpolarized direction relative to the h.-membrane potential relationship. Prior in vitro loose patch voltage-clamp studies on rat and human fast-twitch skeletal muscle fibers from this laboratory (32, 34, 38, 39), from Dr. Walter Stühmer’s laboratory (41), and collaborative studies between this laboratory and Dr. Stühmer’s laboratory (35, 36) found similar separations in the voltage dependencies of fast and slow inactivation for fast-twitch muscle fibers. In vitro studies of Na$^{+}$ channel inactivation in frog twitch skeletal muscle fibers (2) and crayfish giant axons (42) also showed that slow inactivation developed at hyperpolarized potentials compared with fast inactivation. In contrast, in studies of cloned skeletal muscle Na$^{+}$ channels, expressed in a variety of nonmuscle cell systems, the operating voltage ranges of fast and slow inactivation overlapped (10, 13, 16, 17, 29, 43, 46). Consequently, when skeletal muscle Na$^{+}$ channels were expressed in nonmuscle cells, the operating voltage ranges of fast and slow inactivation were not distinct, whereas, when skeletal muscle or nerve Na$^{+}$ channels were studied in the native tissue, the voltage dependence of slow inactivation was shifted in a hyperpolarizing direction compared with fast inactivation.

The causes for the differences in the relative voltage dependencies of fast and slow inactivation of Na$^{+}$ channels studied in expression systems compared with native tissues are not known. However, precedents exist for variations in the voltage-dependent behavior of skeletal muscle Na$^{+}$ channels based on the cell in which the Na$^{+}$ channel was expressed. Na$^{+}$ channels are formed from the same glycoprotein in fast- and slow-twitch fibers (20). Several laboratories reported differences in the voltage dependencies of Na$^{+}$ channel activation and inactivation between mammalian fast- and slow-twitch skeletal muscle fibers (12, 21, 34–36, 41). In mammalian slow-twitch fibers, slow inactivation develops at hyperpolarized potentials compared with fast inactivation; however, when studied at room temperature, the separation between the fast and slow inactivation curves is smaller for slow-twitch fibers compared with fast-twitch fibers (34–36, 38, 39). It is not known why the voltage dependencies of activation and fast and slow inactivation differ between mammalian fast- and slow-twitch muscle fibers (34).

This study showed that, at a physiological temperature of 37°C, most skeletal muscle Na$^{+}$ channels in type IIb fibers were excitable. Comparing type IIa fibers with type IIb mammalian skeletal muscle fibers, $V_{\text{h},2}$ was similar and $V_{\text{h},2}$ was positively shifted for type IIa
fibers (34, 39). Both fast and slow inactivation developed at more positive potentials for type I fibers compared with type IIb fibers (34, 38). Consequently, most Na^+ channels on type I and IIa skeletal muscle fibers should be excitable at the resting potential at 37°C.

This study and prior studies of slow inactivation of I_{Na} in mammalian skeletal muscle fibers from rabbits (21), rats (32, 34), and humans (34, 38, 39) found that slow inactivation could eliminate I_{Na}. In addition, Na^+ channels were completely susceptible to slow inactivation in frog twitch muscle fibers (2) and crayfish axons (42). Cummins and Sigworth (10) reported that slow inactivation could reversibly eliminate I_{Na} for rat skeletal muscle Na^+ channels (rSkM1, µ1) expressed in HEK cells. Conversely, in some studies of Na^+ channels expressed in nonmuscle cell lines, slow inactivation was incomplete, with ~15–20% of I_{Na} not affected by slow inactivation (13, 16, 29, 43). In some studies, slow inactivation would eliminate I_{Na} only if the expressed Na^+ channels had impaired fast inactivation (13, 43). The findings in this study are compatible with those of Cummins and Sigworth (10) for rSkM1 Na^+ channels expressed in HEK cells and with the findings from crayfish axons and muscle fibers from frogs, rabbits, rats, and humans.

Skeletal muscle membrane excitability is a complex interplay of Na^+, K^+, and Cl^- conductances. In cold environments, extremity skeletal muscle cools to conserve core body temperature. Reduction of the population of excitable Na^+ channels at lower temperature may help to prevent skeletal muscle membrane hyperexcitability. At physiological temperatures, Na^+ channels open slightly faster than delayed rectifier K^+ channels. However, at 37°C, mammalian skeletal muscle delayed rectifier K^+ channels activate sufficiently rapidly to assist in terminating the action potential (44). When activation is modeled according to Hodgkin-Huxley kinetics (18, 19), the time constants for the activation parameter for Na^+ channels, \tau_m, and for delayed rectifier K^+ channels, \tau_n, both decrease with increasing temperature. The Q_{10} values for the decline of \tau_m and \tau_n with temperature have been reported as similar in rat skeletal muscle (4). The activation rate for Na^+ channels varied as \tau_m^{-3} and the activation rate of delayed rectifier K^+ channels varied as \tau_n^{-4}. Therefore, reduced temperature produced a greater slowing of activation for delayed rectifier K^+ channels compared with Na^+ channels. Consequently, as the temperature of skeletal muscle declined, the difference in the rate of activation of Na^+ channels and delayed rectifier K^+ channels increased. At 23°C, mammalian skeletal muscle delayed rectifier K^+ channels opened too slowly compared with Na^+ channels to affect action potential termination (1). Consequently, reducing the number of excitable Na^+ channels at lower temperatures may compensate for the slower opening of delayed rectifier K^+ channels and allow the muscle membrane to maintain an appropriate level of excitability.

Possible roles of slow inactivation in inherited clinical disorders of skeletal muscle membrane excitability. Clinical disorders of skeletal muscle membrane excitability are caused by mutations of SCN4A, the gene for the adult form of the human skeletal muscle Na^+ channel (3, 6, 31). Hyperkalemic periodic paralysis (HPP) is an autosomal dominant disorder characterized by attacks of weakness that are commonly associated with elevated serum K^+ levels. Myotonia was also present in some families with HPP. Paralytic attacks in HPP may be induced by K^- loading, cold environment, or rest after exercise. Several point mutations in SCN4A are associated with HPP. Two mutations, Thr704Met and Met1592Val, account for ~90% of the genotyped kindreds. Studies on muscle biopsies showed that paralysis resulted from membrane depolarization that rendered the membrane inexcitable due to inactivation of Na^+ channels. A persistent I_{Na} caused the depolarization (31). Single-channel studies of mutant Na^+ channels demonstrated persistent I_{Na}, resulting from disrupted fast inactivation with an excessive amount of slow mode Na^+ channel gating (7, 15, 17) and window currents created by a membrane potential range over which a small fraction of Na^+ channels could open and not fact inactivate (10, 47). Window currents were created by shifts in the steady-state voltage dependence of activation (10) or both activation and fast inactivation (47). For the pathological persistent I_{Na} to last >10 s, the mutations producing HPP could disrupt slow inactivation (10, 33). Mutations associated with myotonia may have altered slow inactivation, enabling a paralyzing persistent I_{Na} to exist (17, 29). Alternatively, the mutations would not have to involve slow inactivation if slow inactivation was always incomplete so that a fraction of I_{Na} was not subject to slow inactivation (16, 17).

Slow inactivation was disrupted in two of four studied Na^+ channel mutations associated with HPP, including the most common mutations Thr704Met and Met1592Val (10, 16, 17). Two mutations, Met1360Val and Ala1156Thr, displayed the same pattern of slow inactivation of I_{Na}, as wild-type channels when studied at room temperature (16, 17). The slow inactivation results for the Met1360Val and Ala1156Thr mutations can be reconciled in two ways. First, as discussed above, slow inactivation may be incomplete in vivo so that slow activation inactivation need not be disrupted for mutant Na^+ channels to produce persistent depolarizing I_{Na} (16, 17). The second way of reconciling the Met1360Val and Ala1156Thr slow inactivation data is that these mutations may have abnormal temperature dependencies for slow inactivation that would reduce the impact of slow inactivation at physiological temperatures but not at room temperature. For example, the voltage dependence of slow inactivation of the mutant channels at 37°C could have a positive voltage shift and the mutations could have a larger temperature dependence compared with normal channels. Consequently, at physiological temperatures, larger than usual depolarizations would be required to slow inactive mutant Na^+ channels. Hence, slow inactivation would not
terminate the pathologically persistent $I_{\text{Na}}$. A larger temperature dependence could result in slow inactivation for the mutant Na$^+$ channels being similar to normal channels when studied at room temperature.

Paramyotonia congenita (PC) is an autosomal dominant clinical disorder of skeletal muscle membrane excitability (3, 6, 31). PC overlaps clinically with HPP, and it is associated with mutations of SCN4A. Patients with PC may have myotonia at normal temperature, but the prominent features are 1) myotonia that is aggravated or elicited by skeletal muscle cooling, 2) cold-induced weakness that follows myotonia, and 3) worsening of myotonia by exercise (paradoxical myotonia). Muscle biopsies from PC patients demonstrated normal membrane properties at 37°C but cooling to 27°C triggered depolarization to about −40 mV. A pathologically persistent $I_{\text{Na}}$ caused the depolarization (3, 6, 22, 31). Single-channel studies of mutant Na$^+$ channels usually demonstrated alterations in fast inactivation, but these studies did not explain why pathologically persistent $I_{\text{Na}}$ and paralysis were elicited by cooling. One suggestion for the PC mutations was that mutant channels could directly transit from the fast inactivated state to the open state, thereby enabling a fraction of channels to be open at depolarized potentials (22). However, the hypothesis that depolarized mutant channels would transit between fast inactivated and open channel states does not consider that, unless slow inactivation was perturbed, depolarized mutant channels would accumulate in the slow inactivated state, which would terminate the pathological $I_{\text{Na}}$. Plassart-Schiess et al. (27) found that fast inactivation was not appreciably altered by the Ile693Thr mutation, which was associated with the clinical manifestations of cold-induced weakness without stiffness. They suggested that the cold-induced weakness might result from altered slow inactivation, and Hayward et al. (17) found impaired slow inactivation in the Ile693Thr mutation. A reduction in the temperature dependence or a reversal in the effect of temperature on the voltage dependence of slow inactivation, so that $V_{\text{S12}}$ shifted in a positive voltage direction with cooling, would enhance a cold-induced persistent $I_{\text{Na}}$ by preventing PC mutant channels from entering the slow, inactivated state at reduced temperatures. Normal Na$^+$ channels are more susceptible to slow inactivation with cooling (Fig. 8), which would facilitate the ability of a pathologically persistent $I_{\text{Na}}$ to produce inactivation-induced paralysis.

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